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Bile acids but not acidic acids induce Barrett's esophagus

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ZORA URL: <https://doi.org/10.5167/uzh-118486>

Journal Article

Published Version

Originally published at:

Sun, Dongfeng; Wang, Xiao; Gai, Zhibo; Song, Xiaoming; Jia, Xinyong; Tian, Hui (2015). Bile acids but not acidic acids induce Barrett's esophagus. *International Journal of Clinical and Experimental Pathology*, 8(2):1384-1392.

Original Article

Bile acids but not acidic acids induce Barrett's esophagus

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Received October 22, 2014; Accepted January 17, 2015; Epub February 1, 2015; Published February 15, 2015

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Keywords: Barrett's esophagus, bile acids, esophageal reflux, esophageal adenocarcinoma

Introduction

Esophageal adenocarcinoma (EAC) has been linked to chronic inflammation with an incidence that has increased by greater than 500% [1]. The primary risk factor for EAC is Barrett's Esophagus (BE), which invokes dysplastic progression [2]. Although there is great interest in the process of EAC [3-5], little is known regarding the pathogenesis of BE.

BE is defined as the replacement of squamous epithelium in the distal esophagus with metaplastic intestinal columnar epithelium [6]. Until now, the primary animal model used to study BE has been the rat, comprised of performing an esophagojejunostomy to induce gastroduodenal reflux [7, 8]. The observation that duodeno-esophageal reflux induces BE in rats points to the importance of refluxed contents in BE pathogenesis. Reflux injury to the esophagus results in chronic inflammation, oxidative str-

ess, and DNA damage that may contribute to the metaplastic and dysplastic conversion of BE [9].

Several clinical and experimental studies have shown that reflux of bile acids (BAs) into the esophagus contributes to the induction of esophageal injury, BE, and EAC [10-13]. However, the role of BAs and gastric acids due to gastroduodenal reflux has not been clearly studied. Here in, we aimed to utilize two models of Barrett's-like metaplasia, involving animal surgery and feeding of exogenous chemicals, to provide insight into the pathogenesis of BE.

Methods

Animals and treatment procedures

Eight-week-old male Wister rats weighing 250-280 g (obtained from Shandong University Laboratory Animal Center, Jinan, China) were

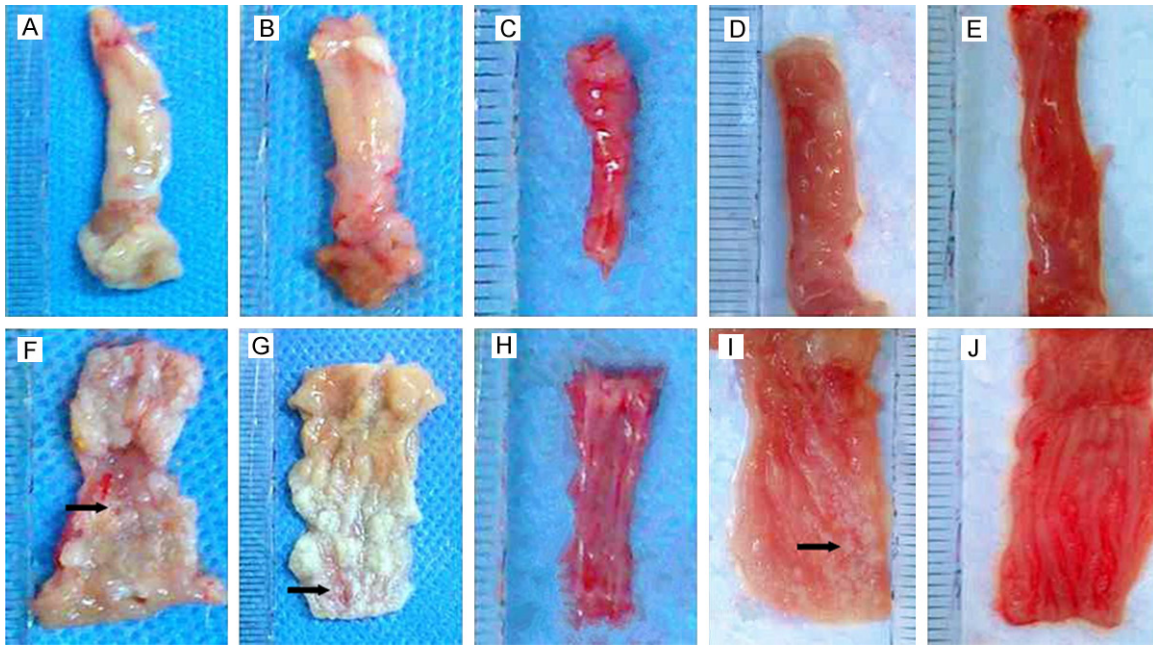


Figure 1. Appearance of esophagus after 6 month of surgery. C/H show the gross appearance of the intact esophagus. A/F and B/G display the appearance of that suffered from 6 months of reflux due to surgical procedures (A/F, duodenoesophageal reflux; B/G, duodenogastroesophageal reflux). Surgical group rats show abnormally dilated esophagus, and esophageal inner surface displays nodular patches (arrow in G) and ulcers (arrow in F). D/I and E/J show the esophagus of animals fed with exogenous chemicals (D/I, bile acids feeding; E/J, acidic water feeding). The mucosa of these two groups become thick and nodular patches occur (arrow in G).

used in this study. All animal handling was carried out by experienced biotechnicians. The rats were housed under standard laboratory conditions with a 12 h light-dark cycle with three animals per cage. Animals were fed commercial rat chow with water provided ad libitum, without exposure to carcinogens. They were allowed to acclimate at least 1 week prior to surgery. Animals were weighed on a weekly basis. Solid food was withdrawn the day prior to surgery and for 1 day after surgery. For the following 24 hours, rats were fed with 10% glucose saline, and then with a regular diet.

Experiment I: surgical model

In group A, 30 rats underwent an end-to-side esophagojejunostomy with gastrectomy, designed to result in duodenoesophageal bile reflux. Briefly, premedication with diethyl ether was performed in a closed induction box prior to positioning on the operating table. A midline laparotomy was performed, during which the esophagus was transected at the esophago-gastric junction and anastomosed end-to-side to the jejunum with a single-layer, interrupted 7-0 prolene suture. Approximately 6-8 stitches were necessary to ensure adequate mucosa-to-mucosa apposition. The stomach was res-

ected and the proximal duodenum was sutured with plasma muscularis embedding. By this method, duodenoesophageal reflux (without gastric acid) was established. The abdomen was irrigated with ceftriaxone sodium (160 mg/kg) prior to closure. Intraoperatively, a simple device containing diethyl ether was used to maintain depth of anesthesia. Immediately postoperatively, 2 mL of 10% glucose was administered subcutaneously.

In group B, 30 rats underwent side-to-side esophagogastrorjejunostomy without gastrectomy. Briefly, after median laparotomy, the proximal jejunum was anastomosed in a side-to-side fashion with the distal esophagus for an anastomotic diameter of 1 cm. A one-layer interrupted 7-0 prolene suture was used to perform the anastomosis. To avoid anastomotic bleeding, some gastric vessels required ligation. The remaining process was similar to that of group A. In this manner, duodenogastroesophageal reflux was induced.

Group C was the control group, in which ten rats underwent median laparotomy. After abdominal closure, these rats also received 2 mL of 10% glucose subcutaneously.

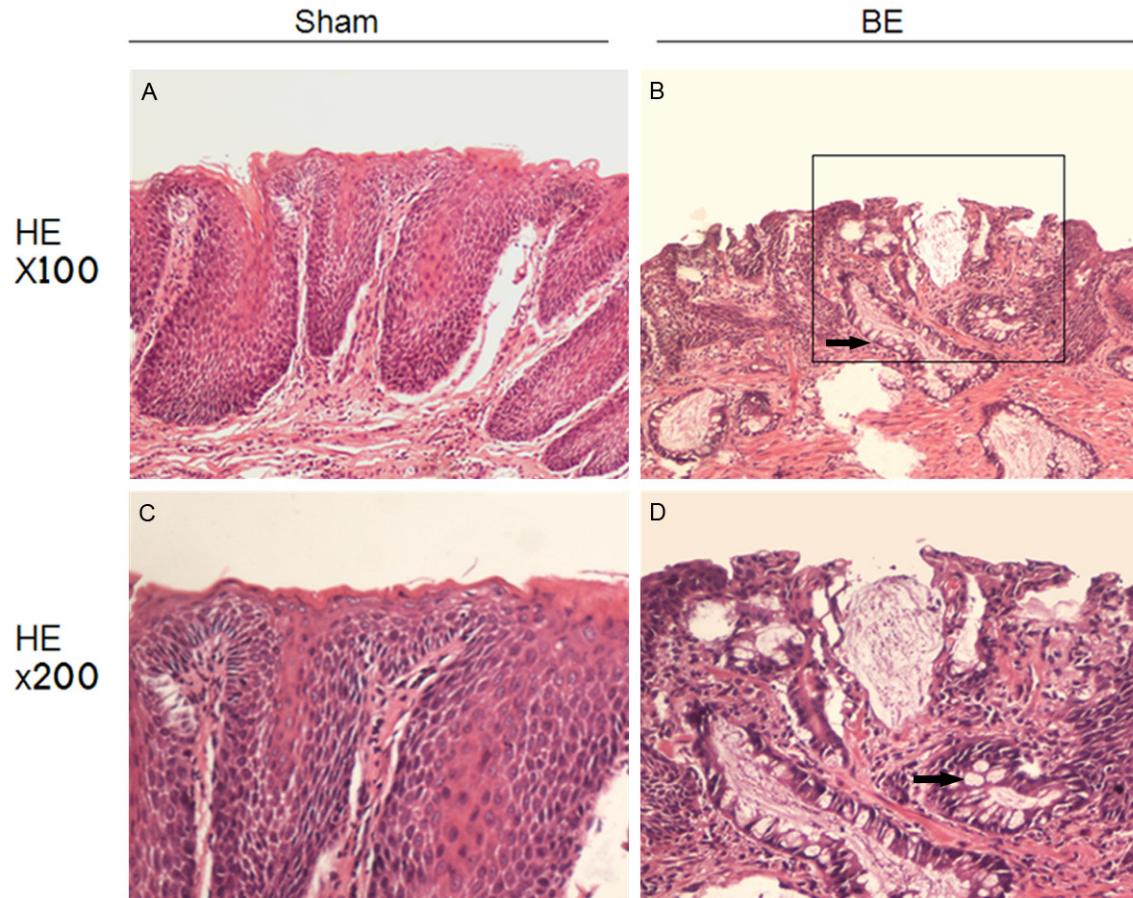


Figure 2. Histopathology of esophageal. The sham group A/C show the normal squamous epithelium. Barrett's epithelium, B/D intestinal type mucosa is present and bound on either side by squamous epithelium. Arrows indicate goblet cells in metaplastic epithelium.

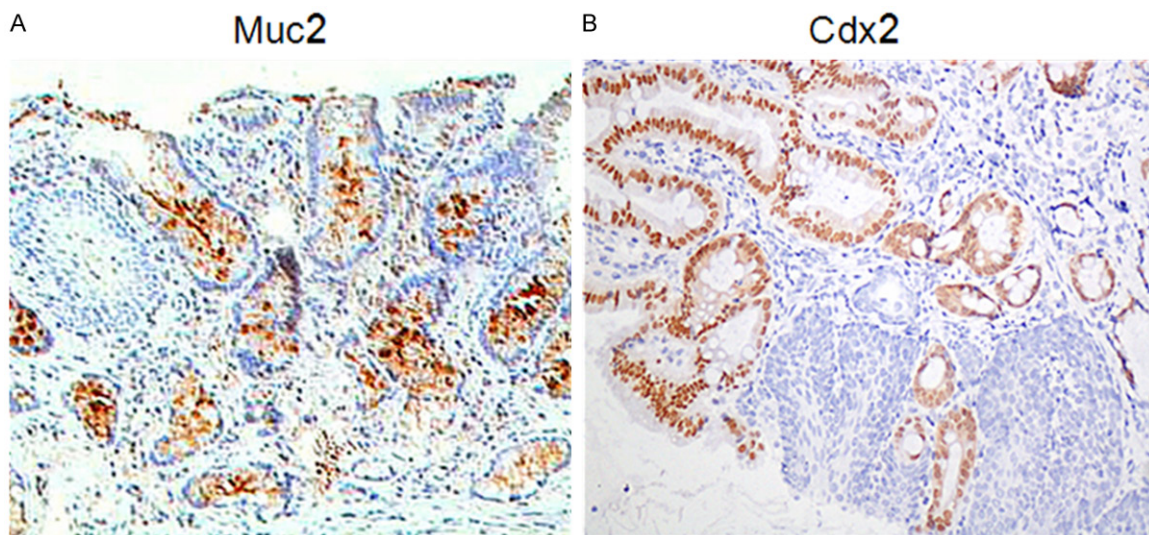


Figure 3. Representative results of Muc2 and Cdx2 immunohistochemical staining on esophageal specimens. A. Marked positive staining for Muc2 (brown) in the glandular component of BE (200 ×). B. Expression of Cdx2 (brown) in the nuclei of BE areas (200 ×).

Bile acids induce Barrett's esophagus

Table 1. Histopathological findings after 6 months of surgery

Histopathologic Findings	Group A (esophagoje junostomy) (n = 17 + 3)	Group B (esophago-gas trojejunostomy) (n = 24 + 2)	Group C (control) (n = 10)
Hyperkeratosis	11	21	0
Squamous hyperplasia	9	17	0
Esophagitis	13+3	19	0
Ulceration	5	4	0
Barrett's esophagus	10 (50%)	12 (46.1%)	0
Carcinoma	3	5	0

No obvious difference was found in the incidence of esophageal disease between group A and B.

Table 2. Histopathological findings of animals fed with exogenous chemicals

Histopathologic Findings	Group D (Bile acid feeding) (n = 29)	Group E (Acidic water feeding) (n = 30)	Group F (control) (n = 10)
Hyperkeratosis	18	15	0
Squamous hyperplasia	15	11	0
Esophagitis	20	9	0
Ulceration	3	2	0
Barrett's esophagus	6 (21.4%)	0	0
Carcinoma	0	0	0

Single asterisk indicates significant difference in the incidence of esophagitis between group D and E ($\chi^2 = 8.96$, $P < 0.01$). Double asterisks indicate significant difference in the incidence of Barrett's esophagus between group D and E ($\chi^2 = 4.83$, $P < 0.05$).

All three groups of rats were sacrificed 6 months after surgery.

Experiment II: nonsurgical model

Seventy male Wister rats were randomly divided into three groups (groups D, E, and F). In group D, 30 rats were fed with water and chow containing bile acids (0.3% DCA, pH 7.0). In group E, 30 rats were fed with chow and acidic water containing HCl (0.01 M HCl, pH 2.0) and a physiologic concentration of pepsin (0.5 mg/l HCl; HCl/P). Group F was the control group, in which ten rats were fed with chow and clean water. Animals were fed for 6 months and were sacrificed at the end of the experiment.

Histologic examination

Animals were sacrificed under general anesthesia and the thoracic and abdominal cavities were inspected. The esophagus was removed,

opened longitudinally, examined macroscopically, and divided into three parts. One portion was fixed in 10% neutral buffered formalin, paraffin embedded, and stained with hematoxylin and eosin (H&E). The other two parts were stored at -70°C for subsequent biochemical assays. The slides were separately reviewed by two experienced pathologists, without knowledge of treatment group assignment. The esophagus was examined for the presence of hyperkeratosis, squamous hyperplasia, esophagitis, ulcerations, metaplasia, and carcinoma. Barrett's-type mucosa was defined as intestinal-type mucosa with goblet cell metaplasia, bound on both proximal and distal ends by squamous mucosa. The slides were scored based upon the presence or absence of Barrett's-type epithelium, hyperkeratosis, squamous hyperplasia, esophagitis, ulcerations, and carcinoma.

Determination of plasma IL-6 protein and TNF- α levels

Immediately after experiment termination, blood samples were placed into EDTA-containing vials and used to determine plasma IL-6 and TNF- α concentrations. Blood was centrifuged at 1000 g for 10 min at 4°C and the plasma was stored at -80°C . Plasma levels of proinflammatory cytokines IL-6 and TNF- α were evaluated with a highly sensitive ELISA test (Quantikine HS, R&D Systems, Minneapolis, MN, USA).

Immunostaining for Muc2 and Cdx2

Immunostaining was performed on paraffin sections using a microwave-based antigen retrieval technique. The antibodies used in this study included Muc2 (1:400 dilution, Abcam, Cambridge, MA, USA) and Cdx2 (Biogenex, San Ramon, CA, USA). Sections were treated with an Envision+ DAB kit (Dako, Glostrup, Denmark), according to the manufacturer's instructions.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR (sqRT-PCR) was performed for selected genes. Briefly, 2 μg total

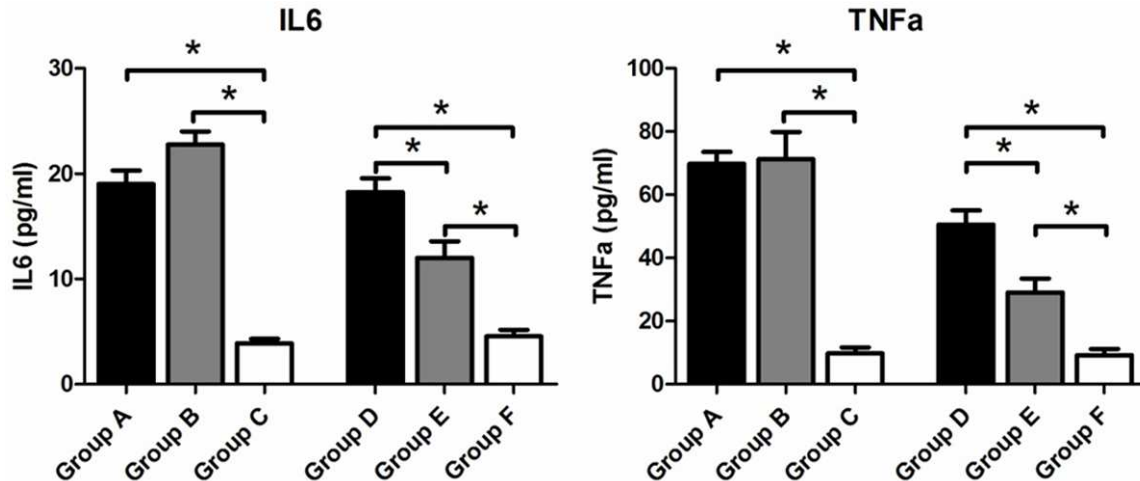


Figure 4. The inflammatory cytokines in the plasma of rats were checked for IL-6 (A) and TNF- α (B). Both surgeries and compounds increased the plasma level of IL-6 and TNF- α . However, no obvious difference between group A and group B in IL-6 and TNF- α levels was observed. Meanwhile, more inflammatory cytokines were observed in group D than in group E. (* $P < 0.05$ data are represented as the mean \pm SEM).

RNA ($n = 5$ to 6) from the same samples used for histologic analysis was reverse transcribed using oligo-dT priming and SuperscriptII (Invitrogen, Carlsbad, CA, USA). First-strand complementary DNA was used as the template for real-time polymerase chain reaction analysis with TaqMan master mix and primers for rMuc2, rCdx2, rBmp4, rKrt19, and rTff2 (Applied Biosystems, Carlsbad, CA, USA). Transcript levels, determined in two independent complementary DNA preparations, were calculated as described and expressed relative to beta-actin as the housekeeping gene.

Statistical analysis

Data are expressed as the mean \pm standard error of the mean. For data regarding sqRT-PCR, the comparison between groups was assessed by one-way ANOVA followed by Bonferroni's test. For data regarding the histologic analysis, the comparison was assessed by using the chi-squared test. Statistical analyses were performed with GraphPad Prism (GraphPad, San Diego, CA, USA) software.

Results

Surgery induces esophagitis, BE, and neoplasia

To understand the pathogenesis of BE, we generated two groups of rats with esophageal reflux, duodenoesophageal reflux, and gastro-

duodenoesophageal reflux. Of the 60 operated animals, 14 (23.3%, ten from group A, four from group B) died prior to the end of the experiment: 1 (7.1%, one from group A) due to an anesthetic accident; three (21.4%, all from group B) from anastomotic bleeding; seven (50%, six from group A, one from group B) due to late anastomotic stricturing; and three (21.4%, all from group A) from generalized weakness. Surviving rats had a poor general outcome: depilation, anemia, and intractable vomiting. However, they all gained weight at the end of the experiment. There were no deaths in the sham-operated group.

Experimental rats showed an abnormally dilated, markedly thickened esophagus in those with gastroduodenal reflux 6 months after surgery, compared with age-matched sham rats. However, there was no difference in the thickness of the BE among surgical groups (**Figure 1A, 1B**). The epithelial surface contained longitudinal folds extending along the lower two-thirds of the esophagus. These findings represented gross evidence of severe esophagitis. Nodular lesions in the lower esophagus were observed in 33 of 46 rats in both surgical groups (**Figure 1F, 1G, 1I**, indicated by arrows). The nodular lesions were associated with carcinoma and basal cell hyperplasia.

Based on previously described criteria, the major histopathologic changes in both surgical groups were inflammation (35 of 46) and pro-

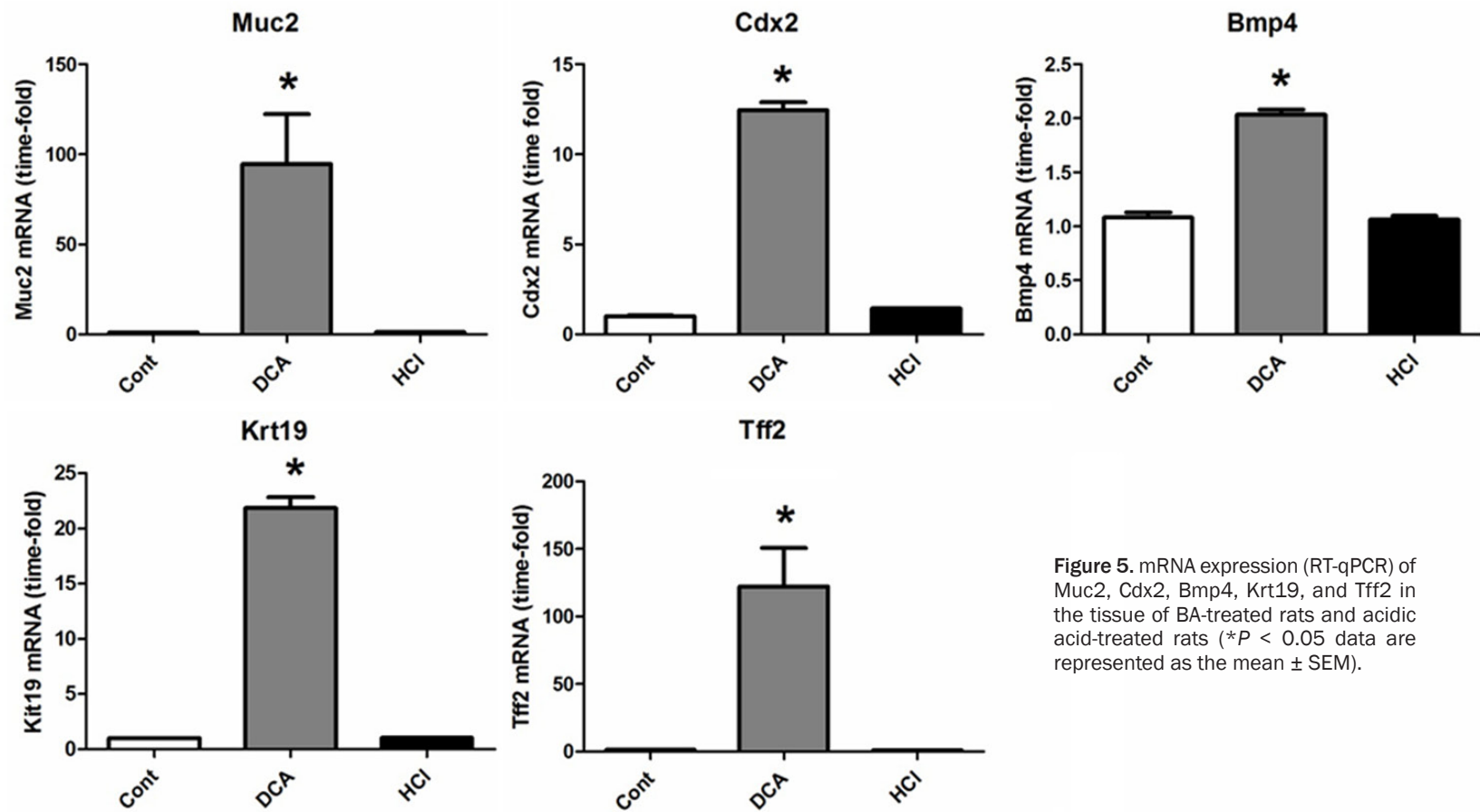


Figure 5. mRNA expression (RT-qPCR) of Muc2, Cdx2, Bmp4, Krt19, and Tff2 in the tissue of BA-treated rats and acidic acid-treated rats (* $P < 0.05$ data are represented as the mean \pm SEM).

found epithelial hypoplasia (26 of 46). Proliferation increased significantly in the basal compartment of the esophagus (**Figure 2B, 2D**).

BE did not occur in control rats; however, 10 of 20 (50%) rats from group A with duodeno-esophageal reflux and 12 of 26 (46.2%) rats in group B with duodenogastroesophageal reflux developed columnar metaplasia with mucus producing (Muc2+) cells (**Figure 3A**), similar to human BE. Glandular metaplasia originated from the lower esophagus because all lesions were above the esophageal anastomosis and had an intact muscularis propria layer on histology. A total of 15% (3/20) of duodeno-esophageal reflux rats and 12.5% (5/26) of duodenogastroesophageal reflux rats developed HGD and EAC (**Table 1**). These lesions were grossly visible within the distal end of the esophagus. During this stepwise progression to BE, we observed an increased expression of Cdx2 in groups A and B, without positive Cdx2 staining in the sham group (**Figure 3B**).

Bile acids but not gastric acids induce the development of Barrett-like metaplasia

BE and EAC have been attributed to gastric acid and/or BA reflux leading to chronic esophagitis. In our two surgical rat models of esophageal reflux, metaplasia and dysplasia appeared to be more dependent upon bile acid compared with gastric acid exposure; while both surgeries allowed BA reflux, reflux with a mixture of gastric acid did not worsen the pathologic changes of BE ($X^2 = 0.0565$, $P > 0.05$, **Table 1**). To examine the impact of gastric acid and BA separately, we compared the effect of BA and gastric acid by feeding animals with deoxycholic acid (DCA) or acidic water in experiment II.

Seventy animals underwent the experiment (30 each for groups D and E, ten for group F). Sixty-nine (98.6%, 29 from group D, 30 from group E, ten from group F) rats survived at the end of the experiment. The remaining rat died from weight loss and generalized weakness. Histologic examination of the esophagus is shown in **Table 2**.

Acidic water treated rats did not show evidence of Barrett's-like metaplasia. However, BA treated rats showed moderate esophagitis with inflammatory infiltrates (**Table 2; Figure 1D, 1I**).

A total of 20.7% (6/29) of BA-treated rats showed metaplastic changes with higher overall metaplasia scores ($X^2 = 4.83$, $P < 0.05$, **Table 2**), indicating a significant induction by BA of Barrett's-like metaplasia. None of the BA treated rats had microscopically visible tumors in the distal esophagus. These data suggest that BAs play a significant role in the pathogenesis of Barrett-like metaplasia; although, we did not use BA at a pH level of 2, and therefore cannot rule out that gastric acid may also play a role in esophageal carcinogenesis.

Bile acids induce inflammation in BE

Previous studies of BE in animal models suggest that BA-induced inflammation is crucial for the development of metaplasia [14]. BA treatment results in an increase of inflammatory cells in the esophageal tissue (20/29, **Table 2**), but fewer inflammatory cells were found in the acid-treated group (9/30, $X^2 = 8.96$, $P < 0.01$). The same increase of inflammation was shown in esophageal tissues from surgical rats. In addition, we observed elevated IL-6 and TNF- α levels in the plasma of BA-treated rats as well as our two surgical groups of reflux rats (**Figure 4**). Consistent with other studies, we confirmed that BAs contribute to the development of BE by inducing the inflammatory response in the esophagus.

Bile acids induce differential gene expression in BE

Finally, we examined the expression of marked differentiation genes in BE. BA-treated rats harboring metaplasia and reflux rats showed elevated levels of Cdx2 and Muc2, which are consistent with immunostaining results (**Figures 3, 5**). Bmp4, Kit19, and Tff2, which are known to be involved in cellular differentiation and proliferation, were also associated with BA treatment and/or BA reflux (**Figure 5**). These increased gene levels were also confirmed in previous studies [14, 15].

Discussion

Using two surgical models of gastroesophageal reflux and rats treated with BAs, we demonstrated that BAs in reflux is sufficient to induce esophagitis and Barrett's-like metaplasia in the esophagus. However, gastric acid alone is not as effective in introducing BE as BAs. Immu-

nostaining and gene expression provided evidence that our rat models closely resemble characteristics of BE. We conclude that Barrett's-like metaplasia partly results from BA reflux.

Although lesions are induced by acid and pepsin in rabbit-mimicked human reflux esophagitis, the fact that the degree of damage does not completely correlate with the amount of refluxed material, and a considerable number of patients do not achieve complete mucosal healing after treatment with antisecretory medication, suggests other contributing mechanisms in reflux Barrett's-like metaplasia [16]. Mixed reflux has been shown to produce more Barrett's metaplasia in the esophagus than pure acid reflux [12]. Several researchers also found similar results: rats exposed to acid reflux had smaller ulcers than those with mixed reflux [10, 17]. This is also supported by clinical observations that patients with BE experienced significantly prolonged episodes of alkaline reflux, rather than acid reflux with a high content of free bile acids [18]. It could also explain why Barrett's esophagus carries an increased risk of malignant transformation, as prolonged exposure to bile acids has been demonstrated in this group of patients. It is also known that BAs can cause oxidative stress and DNA damage, and can inhibit proliferation of esophageal epithelial cells [19, 20].

Our rat models exhibit a columnar-lined esophagus but lack abundant goblet cells; however, it is increasingly recognized that BE does not require classical intestinal metaplasia to establish the diagnosis [21, 22]. Ten of 20 rats had macroscopic BE in the esophagojejunostomy group and 12 of 26 rats in the esophagogastrorjejunostomy group. Moreover, six of 29 rats had microscopic metaplasia in the BA-treated group compared with none of 30 in the acidic water treated group. This may suggest either a delay in metaplasia development in the BA-treated group, or that other factors (e.g. oxidative stress) are involved in the pathogenesis of Barrett's-like metaplasia. Further study is required to identify the exact effects of BAs on the progression of BE.

EAC is one of the most rapidly increasing cancers in the United States and Europe [1, 23, 24]. As EAC is known to be associated with GERD and BE [25-27], a major issue in the prevention of this cancer is whether a window of

opportunity exists between the diagnosis of reflux-related interstitial metaplasia and the development of adenocarcinoma. Unconjugated bile acids, which are increased in the reflux contents of patients with BE [28] and in patients on a high fat diet [29], accelerated the development of BE and dysplasia. Unconjugated BAs can induce gene promoter demethylation, leading to the activation of IL-6, Cdx2, or Notch 1 gene expression in esophageal cells [30, 31], a finding we can confirm in our BA-treated rats. With BA reflux and/or BA treatment in our rats, there were higher scorings of metaplasia and inflammation, pointing to the possible significance of BAs in the development of BE. Inhibition or saturation of BAs to prevent BA exposure may be a useful treatment of BE or could elongate the window of opportunity.

In summary, based on the finding that surgical experiments and exogenous chemicals induced BE in rat models, we conclude that BAs are involved in the development of Barrett's metaplasia. BAs, but not acidic acids, are a major causative factor for BE in our model and possibly in humans; the prevention of BE by inhibiting BAs requires further investigation.

Disclosure of conflict of interest

None.

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